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Exhibit C

Apolipoprotein(a) stimulates vascular endothelial cell growth and migration and signals through integrin $\alpha V\beta 3$

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Elevated plasma concentrations of Lp(a) [lipoprotein(a)] are an emerging risk factor for atherothrombotic disease. Apo(a) [apolipoprotein(a)], the unique glycoprotein component of Lp(a), contains tandem repeats of a plasminogen kringle (K) IV-like domain. In the light of recent studies suggesting that apo(a)/Lp(a) affects endothelial function, we evaluated the effects of apo(a)/Lp(a) on growth and migration of cultured HUVECs (human umbilical-vein endothelial cells). Two full-length r-apo(a) [recombinant apo(a)] variants (12K and 17K), as well as Lp(a), were able to stimulate HUVEC growth and migration to a comparable extent; 17K r-apo(a) also decreased the levels of total and active transforming growth factor-β secreted by these cells. Using additional r-apo(a) variants corresponding to deletions and/or site-directed mutants of various kringle domains in the molecule, we were able to determine that the observed effects of full-length r-apo(a) on HUVECs were dependent on the presence of a functional lysine-binding site(s) in the apo(a) molecule. With respect to signalling events elicited by apo(a) in HUVECs, we found that 17K treatment of the cells increased the phosphorylation level of FAK (focal adhesion kinase) and MAPKs (mitogen-activated protein kinases), including ERK (extracellular-signal-regulated kinase), p38 and JNK (c-Jun N-terminal kinase). In addition, we showed that LM609, the function-blocking antibody to integrin $\alpha V\beta 3$, abrogated the effects of 17K r-apo(a) and Lp(a) on HUVECs. Taken together, the results of the present study suggest that the apo(a) component of Lp(a) signals through integrin $\alpha V\beta 3$ to activate endothelial cells.

Key words: apolipoprotein A, endothelial cell, kinase phosphorylation, integrin $\alpha V\beta 3$, transforming growth factor-β, wound healing.

INTRODUCTION

Elevated plasma concentrations of Lp(a) [lipoprotein(a)] are currently recognized as an emerging risk factor for the development of a variety of atherosclerotic and thrombotic disorders, including peripheral vascular disease, venous thromboembolism, stroke and coronary heart disease [1]. Lp(a) contains a moiety which is indistinguishable from LDL (low-density lipoprotein), and also possesses the unique glycoprotein moiety apo(a) [apolipoprotein(a)]. Apo(a) exhibits a high degree of homology with plasminogen [2,3] and confers unique functions to Lp(a). In the Lp(a) particle, apo(a) is covalently linked, via a single disulfide bond, to the ApoB (apolipoprotein B-100) component of the LDL-like moiety [4]. Apo(a) contains tandem repeats of a sequence that is very similar to the KIV (kringle IV) domain of plasminogen, followed by sequences that are highly homologous with the KV (kringle V) and protease domains of plasminogen [2]. In apo(a), the plasminogen KIV-like domains are further classified as KIV_1 to KIV_{10} based on amino acid sequence. The KIV_2 domain is present in a variable number of identical repeats, which gives rise to the isoform size heterogeneity of Lp(a); there is a single copy of each of the other nine kringle domains in the apo(a) molecule [5,6]. KIV_{10} possesses a strong LBS (lysine-binding site) that may mediate the lysine-dependent binding of apo(a)/Lp(a) to physiological substrates such as fibrin [7,8]. KIV_{5-8} each harbour a weak LBS; those in KIV_7 and KIV_8 play a key role in the assembly of Lp(a) particles [9,10]. The KV domain of apo(a) has been suggested to mediate the ability of apo(a) to inhibit plasminogen activation and may also contribute to maintaining the conformation of the apo(a) molecule [11].

Both pro-atherogenic and prothrombotic properties of Lp(a) have been reported in *in vitro* and *in vivo* studies [1]. Interestingly, evidence is accumulating to suggest a role for apo(a)/Lp(a) in contributing to endothelial dysfunction including stimulation of expression of adhesion molecules and monocyte chemoattractant activity [12,13], and the induction of cytoskeletal rearrangements which may increase the permeability of the EC (endothelial cell) monolayer [14]. Given the importance of EC migration and proliferation in physiological processes such as angiogenesis and wound healing [15,16], we sought to determine whether apo(a)/Lp(a) could affect EC migration and growth in a cultured cell model. Both apo(a) and Lp(a) have been reported to have effects on EC proliferation and migration. However, the reports have been contradictory, with inhibitory effects observed for a recombinant fragment containing KIV_9, KIV_{10} and KV [17], and for single apo(a) KV [18], and stimulatory effects documented for Lp(a) [19]. The latter has been suggested to involve the FGF (fibroblast growth factor)-2 pathway, although the mechanism has not been elucidated, whereas the inhibitory effects of apo(a) on EC migration and proliferation elicited by isolated kringle domains

Abbreviations used: ε-ACA, ε-aminocaproic acid; apo(a), apolipoprotein(a); ApoB, apolipoprotein B-100; bFGF, basic fibroblast growth factor; CM, conditioned medium; DMEM, Dulbecco's modified Eagle's medium; EBM, endothelial basal medium; EC, endothelial cell; ECGS, endothelial-cell growth supplement; ECL, enhanced chemiluminescence; EGM, endothelial growth medium; ERK, extracellular-signal-regulated kinase; FAK, focal adhesion kinase; FBS, foetal bovine serum; FGF, fibroblast growth factor; HUVEC, human umbilical-vein endothelial cell; JNK, c-Jun N-terminal kinase; KIV domain, kringle IV domain; KV domain, kringle V domain; LBS, lysine-binding site; LDL, low-density lipoprotein; Lp(a), lipoprotein(a); MAPK, mitogen-activated protein kinase; MLE, mink lung epithelial; r-apo(a), recombinant apo(a); SMC, smooth muscle cell; TGF-β, transforming growth factor-β; VEGF, vascular endothelial growth factor.

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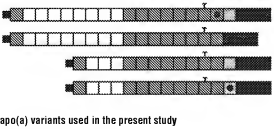

Figure 1 r-apo(a) variants used in the present study

The topology of the r-apo(a) variants used is shown in this schematic diagram. The top line represents the structure of the 17K r-apo(a) variants, which corresponds to a physiological apo(a) isoform and includes all ten types of Kringle IV sequence present in all apo(a) isoforms, as well as the Kringle V (V) and protease-like (P) domains. The black dot within a kringle domain denotes the presence of an amino acid substitution that inactivates the LBS in that kringle. The bar above KIV$_7$ denotes the unpaired cysteine residue in this kringle that mediates covalent attachment to ApoB.

has been reported to involve inhibition of ERK (extracellular-signal-regulated kinase) signalling and FAK (focal adhesion kinase) activation.

In the present study, we first examined the effect of full-length recombinant apo(a), Lp(a) and LDL on cultured EC growth and migration. The results of these assays indicated that the apo(a) component of Lp(a) stimulates both of these cellular responses, which prompted us to study the underlying mechanism by which apo(a) mediates these processes. Our findings demonstrate that apo(a) reduces both total and active TGF-β (transforming growth factor-β) in the cells, and initiates a signalling pathway through the integrin $\alpha V\beta 3$ that results in increased growth and migration of ECs. The results of the present study constitute the first report demonstrating that apo(a)/Lp(a) is able to stimulate EC growth and migration. Moreover, this is the first description of a detailed signalling pathway underlying these apo(a)-specific effects.

MATERIALS AND METHODS

Expression and purification of r-apo(a) [recombinant apo(a)] variants

The r-apo(a) variants used in the present study are shown schematically in Figure 1. The construction and expression of these r-apo(a) variants has been described previously [9,20–22] with the exception of 12KΔLBSV. This variant was obtained by site-directed mutagenesis of the 12K apo(a)-encoding vector using a PCR-based QuikChange® mutagenesis kit (Stratagene). The following oligonucleotides were used for the mutagenesis: 5′-CTGCCGTAACCCTGATGGTGCCATCAATGGTCCCTGGTGC-3′ (sense) and 5′-GCACCAGGGACCATTGATGGCACCATCAGGGTTACGGCAG-3′ (antisense); the oligonucleotides contained a single base change (indicated in bold), resulting in an aspartate-to-alanine substitution at amino acid position 57 of the KV sequence (the first cysteine residue in the kringle is designated as amino acid position 1). The aspartate residue at this position has been shown previously to be a critical component of the LBS in KV [23].

All r-apo(a) variants were purified from the CM (conditioned medium) of stably expressing HEK (human embryonic kidney)-293 cell lines by lysine–Sepharose affinity chromatography as previously described [21,22]. Protein concentrations for each purified r-apo(a) variant were determined by absorbance measurements at 280 nm (corrected for Rayleigh scattering) using the molecular masses and molar absorption coefficients reported previously [20–22]. The molecular mass and molar absorption coefficient for the 12KΔLBSV variant was assumed to be the same as previously reported for 12K r-apo(a) [21].

All proteins were assessed for purity by analysis on SDS/PAGE using a 4–20 % gradient gel followed by silver staining. Purified proteins were aliquoted and stored at −70 °C prior to use.

Lp(a) and LDL purification

Human Lp(a) was purified from fresh plasma by sequential ultracentrifugation and gel-filtration chromatography as described previously [24]. LDL was purified from fresh plasma by sequential flotation as described previously [25].

Cell culture

HUVECs (human umbilical-vein ECs) were obtained from Clonetics and maintained in EGM (endothelial growth medium)-2 (Clonetics) at 37 °C in 5 % CO_2. Wild-type MLE (mink lung epithelial) cells (kindly provided by Dr Geoffrey Pickering, University of Western Ontario, London, ON, Canada) were maintained in DMEM (Dulbecco's modified Eagle's medium)/F-12 medium (Invitrogen) supplemented with 10 % (v/v) FBS (foetal bovine serum; ICN Pharmaceuticals) and antibiotic solution (10000 i.u./ml penicillin, 10000 μg/ml streptomycin and 25 μg/ml amphotericin B; ICN Pharmaceuticals). DR27 cells (MLE cells lacking TGF-β type II receptors [26]) were maintained under the same conditions as wild-type MLE cells.

Cell-growth assay

HUVECs were used at passages 2–6. A confluent 25 cm^2 flask of cells was dispersed using trypsin/EDTA solution (Clonetics). Cells were resuspended in DMEM (Invitrogen) containing 10 % (v/v) heat-inactivated bovine serum (Invitrogen), 50 μg/ml ECGS (endothelial-cell growth supplement, also known as endothelial mitogen; Biomedical Technologies) and 50 μg/ml heparin (Sigma). Approx. 10000 cells in 0.5 ml were added to each well of 24-well tissue culture plates and were allowed to adhere for 24 h. The medium in each well was replaced with 0.5 ml of fresh DMEM supplemented with 5 % (v/v) heat-inactivated bovine serum, 50 μg/ml ECGS and 50 μg/ml heparin. Different r-apo(a) variants were added to triplicate wells at concentrations ranging from 0 to 250 nM. Lp(a) was added to wells at a final concentration of 10 nM; LDL was added at a final concentration of 100 nM. bFGF (basic FGF; PeproTech) at a final concentration of 3 ng/ml was used as a positive control. ε-ACA (ε-aminocaproic acid; Sigma) was added at a final concentration of 10 mM to wells in the absence or presence of either the r-apo(a) variants or Lp(a). LM609 (anti-integrin $\alpha V\beta 3$; kindly provided by Dr David Cheresh, The Scripps Research Institute, La Jolla, CA, U.S.A.) was added to wells to a final concentration of 4 μg/ml, as previously described [27], in the absence or presence of either 17K r-apo(a) or Lp(a). TGF-β-neutralizing antibody (Sigma) was added to wells at a final concentration of 30 μg/ml. In all cases, with the exception of the Lp(a) treatment, after 72 h incubation [48 h for Lp(a) treatment], cells were trypsinized and then counted using a Coulter counter.

Wound-healing assay

Wounding of ECs was performed essentially as previously described [28]. Briefly, HUVECs at passages 2–6 were seeded as confluent monolayers in 12-well tissue culture plates (5×10^5 cells/well). The monolayers were incubated in DMEM containing 5 % (v/v) heat-inactivated bovine serum, 50 μg/ml ECGS and 50 μg/ml heparin for 16 h and wounded by scraping

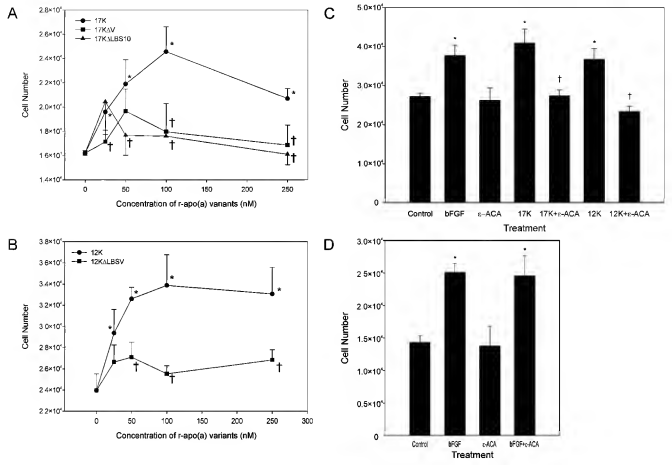

Figure 2 Apo(a) stimulates HUVEC growth

(**A** and **B**) HUVECs were incubated for 72 h with different concentrations of the indicated r-apo(a) variants, at which time the cells were counted. (**C** and **D**) HUVECs were incubated for 72 h with the indicated treatments, at which time the cells were counted. Cells were incubated in the absence (control) or presence of the following: bFGF (3 ng/ml), ε-ACA (10 mM), 17K r-apo(a) (17K; 100 nM), 17K r-apo(a) (100 nM) plus ε-ACA (10 mM), 12K r-apo(a) (12K; 100 nM), 12K r-apo(a) (100 nM) plus ε-ACA (10 mM), or bFGF (3 ng/ml) plus ε-ACA (10 mM). In all cases, the total cell number for each condition was determined by counting the cells using a Coulter counter. The cell number for each treatment was determined from triplicate wells. Values for the cell number correspond to the mean ± S.D.; results shown are representative of three independent experiments. $^{*}P < 0.05$ compared with control and $\dagger P < 0.05$ compared with 17K or 12K, as appropriate.

with a pipette tip. The wounded monolayers were then washed twice with PBS to remove cell debris and incubated in DMEM containing 2 % (v/v) heat-inactivated bovine serum, 50 μg/ml ECGS and 50 μg/ml heparin. r-apo(a) variants [12K and 17K r-apo(a)] and LDL were added to the wells at a final concentration of 100 nM; Lp(a) was added to the wells at a final concentration of 10 nM. In some experiments, either ε-ACA (10 mM) or LM609 (4 μg/ml) was also added to some wells in the absence or presence of either r-apo(a) variants or Lp(a). The rate of wound closure was observed over a 6–8 h period. Light microscopy images of the wounds were obtained using a digital camera, and the width of the wounds was measured. The wound-healing effect was quantified as the remaining cell-free area, calculated by dividing the width of the wounds at each time point by the width of the initial wounds.

TGF-β bioassay

CM from the HUVEC proliferation assay (see above) was harvested at the end of the 72 h incubation period. The quantity of TGF-β in the CM was determined using the MLE cell proliferation inhibition assay as previously described [29]. Briefly, MLE cells and DR27 cells in exponential growth phase were split into 96-well tissue culture plates (10000 cells/well) in 100 μl of medium and allowed to adhere for 2 h. Cells in triplicate wells were treated with 50 μl of CM or TGF-β (R&D Systems) (ranging from 0 to 25 ng/50 μl) and incubated for a further 21 h. To determine the total amount of TGF-β (latent plus active), the CM was acidified with 1 M HCl to a final concentration of 0.167 M for 10 min, followed by neutralization with the same volume of 1 M NaOH. The cells were then pulsed with [^{3}H]thymidine (1 μCi/well; PerkinElmer) for 4 h. Thymidine incorporation was determined by automated harvesting of cells on to glass fibre filters and counting in a scintillation counter. A standard curve for TGF-β concentration was generated and the TGF-β concentrations of all of the experimental samples were determined from this standard curve; values were standardized to cell numbers obtained from the EC proliferation assay described above.

Analysis of signal transduction pathways initiated by apo(a) treatment of HUVECs

HUVECs at passages 2–6 were seeded in six-well tissue culture plates (5×10^4 cells/ml; 3 ml/well) and cultured in EGM-2 for approx. 3 days until they reached confluency. Cells were washed once with PBS and then starved for 3 h in EBM (endothelial basal medium)-2 supplemented with 0.1 % FBS. At the end of the 3 h starvation period, cells were treated with either 17K r-apo(a) at a

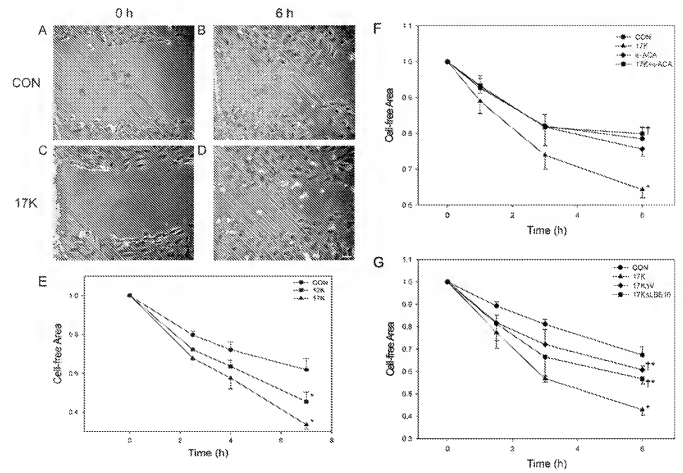

Figure 3 Apo(a) stimulates HUVEC migration

Photomicrographs showing cells after initial scraping (**A**) and migration of cells after 6 h in the absence of 17K r-apo(a) (**B**) (CON); cells after initial scraping (**C**) and migration of cells after 6 h in the presence of 100 nM 17K r-apo(a) (**D**) (17K). Scale bar = 65 μm. (**E**–**G**) Graphical representations of cell migration in the absence (CON) or presence of the indicated treatments [100 nM 17K r-apo(a) (in the absence or presence of 10 mM ε-ACA), 100 nM 12K r-apo(a), 100 nM 17KΔLBS10 or 100 nM 17KΔV]. The cell-free area (width of the wounds at various time points divided by the width of the initial wounds) at each time point after wounding was determined from triplicate wounds. Values for the cell-free area correspond to the mean ± S.D.; results shown are representative of the results obtained from three independent experiments. *$P < 0.05$ compared with control and †$P < 0.05$ compared with 17K.

final concentration of 100 nM for a series of time points (0, 2, 5, 10 and 30 min) at 37 °C, or with 4 μg/ml LM609 in the absence or presence of 100 nM 17K for different time periods to examine kinase phosphorylation (2–30 min). Cells were then placed on ice and lysed in lysis buffer [20 mM Tris/HCl (pH 7.5) containing 150 mM NaCl, 1 mM EDTA, 1 % (v/v) Nonidet P40, 0.5 % (w/v) sodium deoxycholate and 1 mM sodium vanadate] supplemented with freshly added protease inhibitor cocktail (Sigma) for 5 min. Cells were harvested and insoluble material was removed by centrifugation at 12 000 **g** for 10 min.

The activities of FAK and three subtypes of MAPK (mitogen-activated protein kinase), including ERK, JNK (c-Jun N-terminal kinase) and p38 MAPK, were analysed by Western blotting of total HUVEC lysates with antibodies specific for their respective total and active (phosphorylated) forms. Equal volumes of cell lysates were separated by SDS/PAGE and blotted on to Immobilon-P membranes (Millipore). Blots were probed with the following antibodies: α-ERK rabbit polyclonal antibody (1:500), α-phosphorylated ERK mouse monoclonal antibody (1:200), α-p38 rabbit polyclonal antibody (1:300), α-phosphorylated p38 rabbit polyclonal antibody (1:200), α-JNK mouse monoclonal antibody (1:200), α-phosphorylated JNK mouse monoclonal antibody (1:200), α-FAK rabbit polyclonal antibody (1:1000), α-phosphorylated FAK (Tyr^{925}) goat polyclonal antibody (1:200), and α-phosphorylated FAK (Tyr^{861}) rabbit polyclonal antibody (1:1000). All antibodies were purchased from Santa Cruz Biotechnology, with the exception of the α-phosphorylated FAK (Tyr^{861}) antibody, which was from Biosource. The appropriate HRP (horseradish peroxidase)-conjugated secondary antibody (goat anti-rabbit and bovine anti-goat antibodies were from Santa Cruz Biotechnology; sheep anti-mouse and donkey anti-rabbit antibodies were from Amersham Biosciences), as well as the ECL® (enhanced chemiluminescence) system (Amersham Biosciences) were used for visualization of immunoreactive bands in most cases, while an ECL system (Millipore) was used where required to increase the signal intensity. Densitometry was performed using Corel PhotoPaint, Version 10.

Statistical methods

Comparisons between data sets were performed using a Student's t test (assuming equal variances). Statistical significance was presumed at $P < 0.05$. In some cases where the incubation time in the presence of apo(a) was varied, statistical analysis was

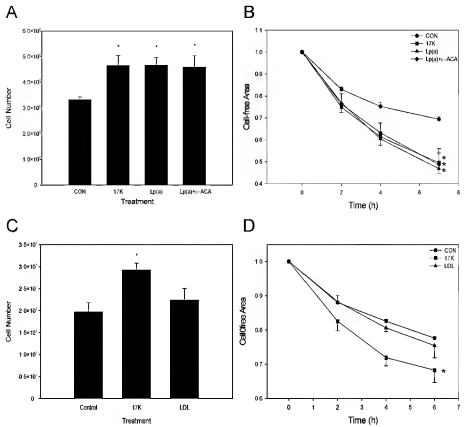

Figure 4 Lp(a), but not LDL, stimulates HUVEC growth and migration

(**A** and **C**) Cells were incubated in the absence (CON) or presence of the following: 17K r-apo(a) (100 nM), Lp(a) (10 nM), Lp(a) (10 nM) plus ε-ACA (10 mM) or LDL (100 nM). Values (mean+S.D.) for each treatment were determined from triplicate wells; results shown are representative of two independent experiments. (**B** and **D**) are graphical representations of cell migration in the absence (CON) or presence of the indicated treatments [100 nM 17K r-apo(a), 10 nM Lp(a), 10 nM Lp(a) plus 10 mM ε-ACA or 100 nM LDL]. The cell-free area (width of the wounds at various time points divided by the width of the initial wounds) at each time point after wounding was determined from triplicate wounds. Values for the cell-free area correspond to the mean ± S.D.; results shown are representative of the results obtained from two independent experiments. *$P < 0.05$ compared with control.

only performed on the data point corresponding to the maximal observed effect.

RESULTS

Apo(a) stimulates HUVEC proliferation and migration

We examined the ability of the recombinant apo(a) variants shown in Figure 1 to modulate the proliferation and migration of cultured human ECs. It was found that both 17K and 12K (corresponding to naturally occurring apo(a) isoforms [30] and containing eight and five copies of the identically repeated KIV_2 sequence respectively) stimulated HUVEC proliferation at all concentrations tested, with the maximal increase in cell number (approx. 1.5-fold) observed at an apo(a) concentration of 100 nM (Figures 2A and 2B). Although results for the 12K and 17K r-apo(a) were comparable, the variants 17KΔLBS10, 17KΔV and 12KΔLBSV (Figure 1), all of which have impaired lysine-binding properties, stimulated HUVEC proliferation to a significantly lesser extent (Figures 2A and 2B). The 17KΔLBS10 and 12KΔLBSV variants each contain the following single amino acid substitutions: aspartate-to-alanine at amino acid position 67 in the KIV_{10} domain for 17KΔLBS10, and aspartate-to-alanine at amino acid position 57 in the KV domain for 12KΔLBSV; these substitutions disrupt the LBS in apo(a) KIV_{10} and KV respectively. 17KΔV is an r-apo(a) variant in which the KV domain has been completely deleted [22]. Taken together, these results indicate that the disruption of the LBS in apo(a) KIV_{10} and KV in the context of full-length apo(a) decreases the stimulatory effects of apo(a) on HUVEC proliferation.

To confirm the importance of the apo(a) LBS in mediating HUVEC proliferation, we used a lysine analogue, ε-ACA, in the cell-growth assay. ε-ACA at a final concentration of 10 mM was added to the cells together with either 17K or 12K r-apo(a) for 72 h. Although ε-ACA addition alone did not result in significant changes in cell numbers, the cell proliferation stimulated by the 17K or 12K was abolished in the presence of ε-ACA (Figure 2C). As a control, ε-ACA was shown to have no inhibitory effect on bFGF-induced HUVEC proliferation (Figure 2D).

In addition to increased mitotic activity, increased motility (i.e. migration) of ECs is an important feature of endothelial cell behaviour observed in processes such as angiogenesis and tumour metastasis. Using a wound healing assay, the two full-length r-apo(a) variants 17K and 12K were both clearly shown to stimulate HUVEC migration into the wounded area at each time point studied (Figures 3A–3E). The ability of apo(a) to stimulate HUVEC migration was not due to its stimulatory effect on HUVEC growth, since under the same experimental conditions as for the wound-healing assay (i.e. with respect to cell seeding density and composition of the medium), 17K r-apo(a) treatment of the cells did not elicit changes in cell

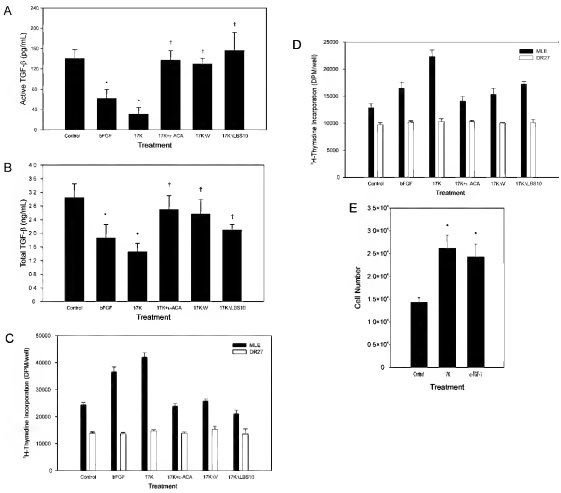

Figure 5 Apo(a) treatment of HUVECs results in the reduction of both active and total TGF-β

HUVECs were incubated for 72 h in the absence (control) or presence of bFGF (3 ng/ml), 17K (100 nM, in the absence or presence of 10mM ε-ACA), 17KΔV (100 nM) or 17KΔLBS10 (100 nM). The concentrations of active (**A**) and total (**B**) TGF-β in the HUVEC CM were determined using a MLE cell bioassay. Values for each treatment were determined from triplicate wells; results shown (means ± S.D.) are representative of three independent experiments. *$P < 0.05$ compared with control and †$P < 0.05$ compared with 17K. (**C** and **D**) The TGF-β bioassay was repeated (with the same HUVEC CM) using a mutant MLE cell line (DR27) which lacks a functional TGF-β receptor. (**C**) [^{3}H]Thymidine incorporation using untreated CM (representing the level of active TGF-β). (**D**) [^{3}H]Thymidine incorporation using acidified CM (representing the level of total TGF-β). The results shown are the raw cell proliferation data; results for each treatment were determined from triplicate wells and the results shown (means ± S.D.) are representative of three independent experiments. (**E**) Cells were incubated for 72 h in the absence (control) or presence of the following: 17K r-apo(a) (100 nM), or a neutralizing antibody against TGF-β (30 μg/ml). Total cell numbers for each condition were determined by counting the cells using a Coulter counter. Values for each treatment were determined from triplicate wells; results shown (means ± S.D.) are representative of three independent experiments. *$P < 0.05$ compared with control.

number after 24 h (results not shown), which is longer than the time frame for the wound healing assay (6–8 h). Consistent with the HUVEC proliferation study, the addition of 10 mM ε-ACA significantly inhibited the stimulatory effect of 17K (Figure 3F) and 12K (results not shown) on HUVEC migration; 17KΔLBS10 and 17KΔV failed to stimulate the wound-healing process to the extent that 17K did (Figure 3G). Since comparable results were obtained for the 12K and 17K r-apo(a) variants in both proliferation and migration assays, only the 17K r-apo(a) was used in all subsequent experiments.

Lp(a), but not LDL, stimulates HUVEC growth and migration

Lp(a) and LDL were tested in both cell-growth and wound-healing assays to confirm that the apo(a) component of Lp(a) mediates these effects. As expected, Lp(a) stimulated both HUVEC growth (Figure 4A) and migration (Figure 4B) to a similar extent as 17K r-apo(a); however, ε-ACA failed to block the stimulatory effects of Lp(a) (Figures 4A and 4B). Nonetheless, since LDL did not stimulate HUVEC proliferation or migration (Figures 4C and 4D), the stimulatory effects of Lp(a) on HUVECs can be attributed to the apo(a) component of Lp(a).

Apo(a) modulates levels of both total and active TGF-β in HUVECs

TGF-β is an important suppressor of EC proliferation and migration [31]. This provided a rationale for investigation of the potential effect of apo(a) on levels of TGF-β in HUVECs. The levels of total and active TGF-β present in the HUVEC CM harvested from apo(a)-treated cells were determined using a TGF-β bioassay that was performed immediately following the cell-growth assay (see above). It was found that the level of active TGF-β was significantly down-regulated in CM harvested from cells treated with a pro-proliferative r-apo(a) variant (17K; Figure 5A), but was unchanged in CM harvested from cells treated with r-apo(a) variants defective in their ability to stimulate HUVEC proliferation (17KΔLBS10 and 17KΔV; Figure 5A). Furthermore, the inhibitory effect of 17K r-apo(a) on the level of

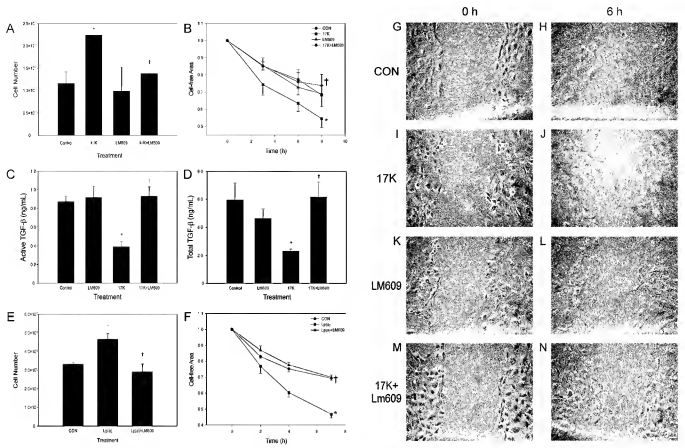

Figure 6 LM609 blocks apo(a)/Lp(a)-induced HUVEC activation

(**A** and **C–E**) HUVECs were incubated for 72 h in the presence or absence of 4 μg/ml LM609 and/or 100 nM 17K r-apo(a) or 10 nM Lp(a). Total cell numbers for each condition were determined by counting the cells using a Coulter counter, and the concentrations of active (**C**) and total (**D**) TGF-β in the HUVEC CM were determined using a MLE cell bioassay as previously described. Values (mean+S.D.) for each treatment were determined from triplicate wells; results shown are representative of three (**A**) or two (**C–E**) independent experiments. (**B** and **F**) Cell migration was studied in the absence or presence of 4 μg/ml LM609 and/or 100 nM 17K r-apo(a) or 10 nM Lp(a). The cell-free area (width of the wounds at various time points divided by the width of the initial wounds) at each time point after wounding was determined from triplicate wounds. Values for the cell-free area correspond to the mean $\pm$ S.D.; results shown are representative of the results obtained from three (**B**) or two (**F**) independent experiments. *$P < 0.05$ compared with control and †$P < 0.05$ compared with 17K (**A**) or 12K (**B**). (**G–N**) Photomicrographs showing cells after initial scraping (**G**) (CON) and migration of cells after 6 h in the absence of 17K r-apo(a) (**H**); cells after initial scraping (**I**) and migration of cells after 6 h in the presence of 100 nM 17K r-apo(a) (**J**); cells after initial scraping (**K**) and migration of cells after 6 h in the presence of 4 μg/ml LM609 (**L**); cells after initial scraping (**M**) and migration of cells after 6 h in the presence of 4 μg/ml LM609 plus 100 nM 17K r-apo(a) (**N**). Scale bar = 65 μm.

active TGF-β was abolished by the addition of 10 mM ε-ACA (Figure 5A). Using these r-apo(a) variants, similar effects on total (i.e. latent plus active) TGF-β were observed (Figure 5B).

Our quantitative analysis of TGF-β concentrations clearly demonstrates that active TGF-β accounts for less than 10 % of the total (latent plus active) TGF-β, whereas an up to 50 % decrease in the levels of total TGF-β was observed in the presence of apo(a). Thus the decrease in total TGF-β cannot be entirely accounted for by the decrease in active TGF-β; clearly, there is a reduction in the amount of latent TGF-β, perhaps owing to modulation of the expression of the gene encoding this growth factor by signalling pathways elicited by apo(a).

The validity of the TGF-β bioassay was further confirmed by repeating the previous experiment using the DR27 strain of MLE cells that lack TGF-β type II receptors [26]. Compared with the results obtained using wild-type MLE cells, the proliferation of DR27 cells (determined by the measurement of thymidine incorporation) remained unchanged in the presence of CM harvested from apo(a)-treated HUVECs (Figures 5C and 5D). This excludes the possibility that apo(a) alters the concentration of a factor in HUVEC CM, other than TGF-β, that accounts for the decreased MLE cell proliferation. Moreover, 17K r-apo(a) was able to stimulate HUVEC growth to a level comparable with that achieved by adding a neutralizing antibody against active TGF-β to the cell medium (Figure 5E). This suggests that the ability of apo(a) to reduce the level of TGF-β is responsible, at least in part, for its stimulatory effect on HUVEC proliferation.

Apo(a) requires functional integrin $\alpha V\beta 3$ to stimulate HUVECs

A previous study has identified a specific interaction between angiostatin, which is structurally analogous to apo(a), and integrin $\alpha V\beta 3$ in ECs [27]. We explored the possibility that this integrin might be the cell-surface receptor that elicits signalling events leading to the activation of HUVECs that we observed upon apo(a) treatment. First, in the cell-growth assay, a function-blocking antibody to integrin $\alpha V\beta 3$ (LM609) was added to the cells in the absence or presence of 17K r-apo(a) and cell proliferation was measured as described above. Although treatment of the cells with 4 μg/ml LM609 alone did not affect cell proliferation, proliferation of HUVECs stimulated by 17K r-apo(a) was completely inhibited by the addition of the same

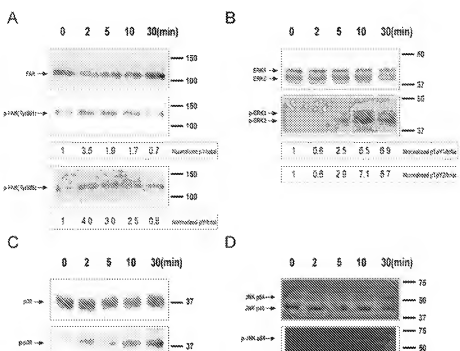

Figure 7 Apo(a) treatment of HUVECs elicits an increase in FAK and MAPK phosphorylation

Confluent HUVECs were serum-starved in EBM-2 plus 0.1 % FBS for 3 h before treatment with 100 nM 17K for 0, 2, 5, 10 and 30 min. The cells were lysed and subjected to SDS/PAGE and Western blot analysis for total, phosphorylated-FAK (Tyr^{861}) [p-FAK(Tyr861)] and phosphorylated-FAK (Tyr^{925}) [p-FAK(Tyr925)] (**A**), total and phosphorylated-ERK1/2 (p-ERK1 and p-ERK2) (**B**), total and phosphorylated-p38 (p-p38) (**C**) and total and phosphorylated-JNK p54 MAPK (p-JNK p54) (**D**). The ratio of phosphorylated-to-total kinase is expressed relative to the ratio observed at 0 min, and is shown as normalized pY (or pTpY)/total in the bottom box. The same pattern of response was observed in three independent experiments. The molecular mass in kDa is indicated on the right-hand side of each gel. pT, phosphorylated threonine; pY, phosphorylated tyrosine.

concentration of LM609 (Figure 6A). This inhibitory effect of LM609 was also observed on 17K-mediated HUVEC migration (Figures 6B and 6G–6N), on 17K-mediated reduction of levels of active and total TGF-β in HUVECs (Figures 6C and 6D), and on Lp(a)-mediated HUVEC proliferation and migration (Figures 6E and 6F).

We have executed several control experiments to demonstrate the specificity of the LM609 antibody in our system. We have plated the cells on collagen, which is not bound by integrin $\alpha V\beta 3$, and found that 17K apo(a) stimulates HUVEC proliferation and that this effect is abrogated by LM609 (results not shown). Furthermore, we have investigated the ability of LM609 to inhibit the proliferation and migration of HUVECs in response to VEGF (vascular endothelial growth factor), whose effects are independent of integrin $\alpha V\beta 3$. As expected, LM609 had no effect on these responses to VEGF (results not shown).

Apo(a) activates FAK phosphorylation

FAK is an indispensable component of an integrin-dependent signalling pathway [32] and can be phosphorylated on multiple tyrosine residues, including Tyr^{397}, Tyr^{576}, Tyr^{577}, Tyr^{861} and Tyr^{925}. Of these residues, phosphorylation of Tyr^{576} and Tyr^{577} is required for maximal FAK catalytic activation [33]. Integrin activation results in auto-phosphorylation of Tyr^{397} of FAK and subsequent recruitment of SFKs (Src-family kinases); Src in turn phosphorylates Tyr^{861} and Tyr^{925} [34]. As such, the Tyr^{861} and Tyr^{925} phosphorylations of FAK are integrin-dependent. In the present study, we treated HUVECs with 100 nM 17K r-apo(a) for various times, and cell lysates were collected. Samples were analysed by Western blotting using α-FAK, α-phosphorylated FAK (Tyr^{861}) and α-phosphorylated FAK (Tyr^{925}) antibodies respectively to measure the extent of FAK phosphorylation on these residues, and thus integrin-dependent FAK activation. Treatment of HUVECs with 17K r-apo(a) resulted in time-dependent increases in the phosphorylation of Tyr^{861} as well as Tyr^{925} (Figure 7A); phosphorylation of both tyrosine residues was maximal at 2 min.

Apo(a) stimulates MAPK phosphorylation

Integrin activation has been shown to initiate signalling through the MAPK pathway; indeed, MAPK has been implicated in a number of cellular processes including cell proliferation and migration [35]. In order to examine the downstream signalling events following the activation of integrin $\alpha V\beta 3$ and FAK, the phosphorylation levels of three MAPKs (ERK, p38 and JNK) were analysed in cell lysates collected from HUVECs that were treated with 17K r-apo(a) for different time periods. Densitometric analyses clearly showed elevated activation of all three MAPKs in HUVECs treated with 17K r-apo(a): ERK1/2 phosphorylation was maximal at 10 min, p38 phosphorylation was maximal at 30 min, and JNK p54 phosphorylation was maximal at either 10 or 30 min (Figures 7B–7D). Notably, although both total JNK p54 and JNK p46 were detected in cell lysates, only

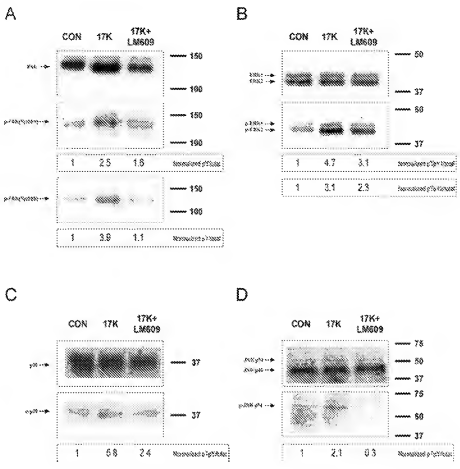

Figure 8 LM609 blocks apo(a)-induced FAK and MAPK activation

Confluent HUVECs were serum-starved in EBM-2 containing 0.1 % FBS for 3 h prior to treatment with 100 nM 17K r-apo(a) (with or without 4 μg/ml LM609) for 2 min (**A**), 10 min (**B**) or 30 min (**C** and **D**). The cells were lysed and subjected to SDS/PAGE and Western blot analysis for total and phosphorylated-FAK [p-FAK(Tyr861) and p-FAK(Tyr925)] (**A**), ERK (p-ERK1 and p-ERK2) (**B**), p38 (p-p38) (**C**) and JNK (p-JNK p54) (**D**). The ratio of phosphorylated to total kinase in each case is expressed relative to the ratio observed for the control [i.e. no 17K r-apo(a)], and is shown as normalized pY (or pTpY)/total in the bottom box. The same pattern of response was observed in three independent experiments. The molecular mass in kDa is indicated on the right-hand side of each gel. CON, control; pT, phosphorylated threonine; pY, phosphorylated tyrosine.

phosphorylated JNK p54 could be detected in our studies (Figure 7D).

Apo(a) requires functional integrin $\alpha V\beta 3$ to activate both FAK and MAPK

To prove that apo(a) signals through integrin $\alpha V\beta 3$ to elicit downstream signalling events, LM609 was used in the kinase activation studies. HUVECs were treated for different times according to the maximal phosphorylation time determined for each specific kinase (2 min for FAK, 10 min for ERK and 30 min for p38 and JNK; see above). Compared with data obtained from the 17K-treated cells in the absence of the antibody, the level of FAK phosphorylation was clearly reduced by the addition of LM609 (Figure 8A); similar results were obtained for ERK, p38 and JNK (Figures 8B–8D).

Lp(a) modulates the level of TGF-β and activates FAK and ERK in HUVECs in an integrin $\alpha V\beta 3$-dependent manner

Both the TGF-β bioassay and kinase phosphorylation assay were performed to test the effects of Lp(a) on TGF-β, FAK and ERK, and whether it is sensitive to LM609 as are those of apo(a). Results consistent with those in Figures 6–8 were obtained, demonstrating that Lp(a) is able to elicit the same cellular responses as apo(a) via integrin $\alpha V\beta 3$, including reducing the level of active and total TGF-β (Figures 9A and 9B), and enhancing the activation of FAK (Figure 9C) and ERK (Figure 9D).

DISCUSSION

Over the past several decades a plethora of mechanisms potentially underlying Lp(a) function and pathogenicity have been uncovered by both *in vitro* and *in vivo* studies. Many studies have revealed effects of apo(a)/Lp(a) on the phenotype of vascular cells including monocytes, macrophages, SMCs (smooth muscle cells) and ECs [1]. However, the signalling pathways underlying these effects remain largely unknown.

In the present study, we initially identified a stimulatory effect of apo(a) and Lp(a), but not LDL, on HUVEC proliferation and migration. This, in turn, suggests a potential role for the apo(a) component of Lp(a) in regulating important physio logical/pathological events associated with altered EC phenotype including angiogenesis, tumour invasion and metastasis, and wound healing. Importantly, a signalling pathway elicited by apo(a) in HUVECs was subsequently dissected and the results suggest a potential role of integrin $\alpha V\beta 3$ as the cell-surface

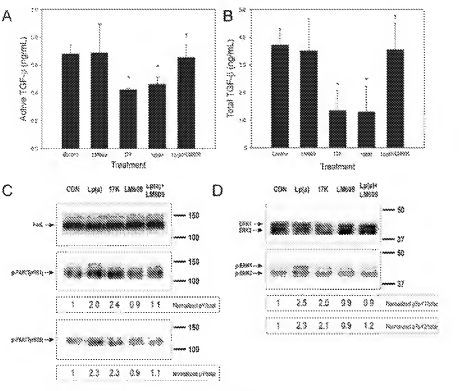

Figure 9 Lp(a) requires integrin $\alpha V\beta 3$ to reduce the level of TGF-β and activate FAK and ERK in HUVECs

(**A** and **B**) HUVECs were incubated for 48 h in the absence (control) or presence of LM609 (4 μg/ml), 17K (100 nM), Lp(a) (10 nM), or Lp(a) (10 nM) plus 4 μg/ml LM609. The concentrations of active (**A**) and total (**B**) TGF-β in the HUVEC CM were determined using a MLE cell bioassay. Values for each treatment were determined from triplicate wells; results (means $\pm$ S.D.) shown are representative of two independent experiments. *$P < 0.05$ compared with control and †$P < 0.05$ compared with Lp(a). (**C** and **D**) Confluent HUVECs were serum-starved in EBM-2 containing 0.1 % FBS for 3 h prior to treatment with 100 nM 17K r-apo(a) or 10 nM Lp(a) (with or without 4 μg/ml LM609) for 2 min (**C**) or 10 min (**D**). The cells were lysed and subjected to SDS/PAGE and Western blot analysis for total and phosphorylated FAK (p-FAK(Tyr861) and p-FAK(Tyr925)) (**C**) and ERK (p-ERK1 and p-ERK2) (**D**). The ratio of phosphorylated-to-total kinase in each case is expressed relative to the ratio observed for the control (i.e. no treatment), and is shown as normalized pY (or pTyr)/total in the bottom box. The same pattern of response was observed in two independent experiments. The molecular mass in kDa is indicated on the right-hand side of each gel. CON, control; pT, phosphorylated threonine; pY, phosphorylated tyrosine.

receptor that mediates the effects of apo(a)/Lp(a) on HUVEC phenotype.

The findings of the present study that apo(a) stimulates HUVEC proliferation and migration are in agreement with the results of Yano et al. [19], but not with those of Schulter et al. [36], who found that apo(a) decreased bFGF-induced HUVEC proliferation. It is notable, however, that an effect of apo(a) on proliferation in the absence of added bFGF was not tested in the latter paper. Inhibitory effects on HUVEC migration and proliferation have also been reported for fragments of apo(a) or apo(a) KV [17,18]; however, these are not physiologically relevant forms of apo(a) and would not be found *in vivo*.

The stimulatory effect of apo(a) on HUVEC proliferation and migration was supported by our observation that apo(a) treatment reduced the levels of both active and total TGF-β; TGF-β has been reported to inhibit EC growth [37–40] and motility [39,41] in cultured cell systems. Interestingly, a previous study by O'Neil et al. [42] reported that apo(a) was able to stimulate human vascular SMC proliferation and migration by reducing the amount of active TGF-β through an indirect mechanism involving apo(a)-mediated inhibition of plasmin formation. The authors did not observe an effect of apo(a) on total TGF-β levels in SMCs [42], which may suggest that the effects of apo(a) on vascular cell behaviour are cell-type-specific. This, in turn, may underscore a role for Lp(a) in a variety of processes involved in atherogenesis.

Our understanding of the mechanism(s) through which apo(a) induces HUVEC growth and migration was greatly facilitated by the availability of a pool of r-apo(a) species with impaired lysine-binding abilities. None of the three mutant r-apo(a) variants (17KΔLBS10, 17KΔV and 12KΔLBSV; each containing a mutation/deletion in the LBS within the KIV_{10} or KV domains) was found to stimulate HUVEC proliferation; 17KΔLBS10 and 17KΔV were unable to stimulate wound healing or to reduce the levels of active and total TGF-β in HUVECs (12KΔLBSV was not tested in the wound-healing assay). Moreover, incubation of cells with the lysine analogue ε-ACA blocked apo(a)-induced increases in proliferation and migration of the cells, and also abolished the effect of apo(a) on TGF-β levels. Intriguingly, in a study by Becker et al. [43], a model was developed suggesting that ε-ACA binds to the strong LBS in apo(a) KIV_{10} and causes apo(a) to shift from a 'closed' to an 'open' conformation. This model was supported by further studies [11,22,44], indicating that the LBS in both apo(a) KIV_{10} and apo(a) KV are required for maintaining the 'closed' conformation of apo(a). Taken together, the results in the present study support a role for the closed conformation of apo(a) in mediating its effects on HUVEC migration and proliferation. However, to our surprise, ε-ACA did not block Lp(a)-induced HUVEC growth or migration. This result may be explained by the inability of ε-ACA at the concentrations used in the present study to allow apo(a) to adopt an open conformation in the context of the Lp(a) particle. This is a reasonable assumption based on the constraints that are probably imposed on apo(a) through its extensive interactions with ApoB in Lp(a) [45,46]. The use of higher concentrations of ε-ACA was not possible in the present study due to effects on cell viability (results not shown).

Following the observation of the effects of apo(a) and Lp(a) on HUVEC phenotype, we demonstrated a novel signalling pathway that apo(a) elicits in HUVECs, starting from integrin $\alpha V\beta 3$ to FAK and advancing to MAPK. Integrins are key players in the development and integrity of the cardiovascular system reflecting their involvement in cell adhesion and migration, cell-cycle progression, cell differentiation and apoptosis [47]. The present study demonstrates that LM609, the function-blocking antibody to integrin $\alpha V\beta 3$, blocks all of the observed effects of apo(a)/Lp(a) on HUVECs, including the effects on cell proliferation, migration, level of TGF-β, and FAK and MAPK phosphorylation. Notably, a previous study has shown that the blockade of integrin $\alpha V\beta 3$ impaired HUVEC growth and survival [48]. Comparison of direct with indirect effects of apo(a) on integrin $\alpha V\beta 3$ signalling will be an important next step in our studies.

Integrins recruit FAK through their β-subunits and the role of FAK signalling in the control of cell survival, proliferation and motility has been extensively reviewed [33,49]. We observed an elevated phosphorylation level of the Tyr^{861} and Tyr^{925} residues of FAK in HUVECs upon treatment with apo(a). The phosphorylation of FAK at Tyr^{861} is believed to recruit p130Cas which acts through either Rac or B-Raf to activate MAPK; the phosphorylation of FAK at Tyr^{925} would recruit GRB2 (growth factor receptor-bound protein 2) binding to FAK and thus activate the Ras-MAPK cascade [34,50]. In the present study, apo(a)-dependent FAK activation (2 min) temporally preceded the activation of ERK, p38 and JNK, with ERK activation (10 min) occurring prior to the activation of p38 and JNK (30 min). These results agree well with the arrangement of the proposed signalling pathway [34], in that FAK is upstream of MAPK. MAPKs have profound effects on almost all aspects of cell behaviour; a vast array of ERK, p38 and JNK downstream targets are involved in cell proliferation and migration. Of particular interest to the present study, integrins have been reported to be strong activators of JNK compared with most growth factors [51], and JNK itself has been reported to repress TGF-β gene expression [52], which may explain the effect of apo(a) on TGF-β levels that we observed.

Although the present study strongly suggests a role for integrins in apo(a) signalling in ECs, it does not exclude the possibility that apo(a) also interacts with other membrane receptors to elicit its downstream effects. For example, it has been shown that, to stimulate cell proliferation, the combined activation of integrins and RTKs (receptor tyrosine kinases) is necessary to activate Ras-ERK signalling beyond the threshold required for transcription of cyclin D [53]. Additional studies will also be required to understand whether or not apo(a) interacts directly with integrins on ECs. Interestingly, an RGD sequence is present in apo(a) KIV_8 [2]. A recent study using an RGDS peptide has suggested that the RGD sequence in apo(a) binds to the IIb subunit of the fibrinogen receptor, thereby inhibiting platelet aggregation [54]. Direct examination of the role of this sequence in mediating apo(a)–integrin interactions is clearly warranted. Additionally, the functional ramifications of the stimulatory effect of apo(a) on the processes of EC migration and proliferation should be studied using appropriate *in vivo* models.

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